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Real-time quantitative PCR detection of *Mycobacterium avium* subsp. *paratuberculosis* and differentiation from other mycobacteria using SYBR Green and TaqMan assays

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Abstract

Sensitive real-time sequence detection methods based on two different chemistries were developed for *Mycobacterium avium* subsp. *paratuberculosis* (Map), the causative agent of Johne's disease in cattle. One is based on the detection of SYBR Green bound to PCR products and the second method is more specific, detecting the cleavage of a fluorogenic (TaqMan) probe bound to a target sequence during primer extension phase. Novel primers and probes that amplify small fragments (<80 bp) of the Map specific insertion sequence, IS900 were designed. Both the SYBR Green and TaqMan assays are sensitive, able to detect 4 fg of DNA extracted from Map strain ATCC19698. This amount of DNA corresponds to the detection of 0.8 cells. Map cells were quantified directly from 7H9 broth using the SYBR Green assay and compared to dilutions of DNA extracted from an equivalent number of cells. The SYBR Green assay of 7H9 broth resulted in a minimum detectable limit of 0.07 cells (equivalent to 0.34 fg of DNA). Media ingredients were not observed to interfere with the assay. Since no extraction step was necessary in the direct cell measurements, direct detection was ten-fold more sensitive than detection of extracted DNA. Both SYBR Green and TaqMan assays are highly specific for the detection of Map. They did not detect any closely related members of the avium complex, other species of mycobacteria, or related genera that are likely to be present in environmental samples. No reporter signal was detected during TaqMan assays performed with 100 pg of template DNA from the non-Map organisms.

Keywords: Real-time PCR; TaqMan; Quantitation; Detection; SYBR Green; *Mycobacterium avium* subsp. *paratuberculosis*

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (Map), the causative agent of Johne's disease in cattle, is responsible for a $250 million annual loss

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to the US dairy industry (Ott et al., 1999). Such losses can be minimized by early detection and culling of infected cattle and by preventing the oral–fecal routes of transmission, as the pathogen survives in feces for longer than a year (Whittington et al., 2004). Early detection of infection is difficult since the fecal culture methods, the current gold standard, can take as long as 4–6 months to confirm the presence of pathogens.

Map is known to be associated with Crohn's disease in humans and the evidence to support the possible causation of Crohn's by Map is growing rapidly (Bull et al., 2003; Chamberlin et al., 2001; Greenstein, 2003; Hulten et al., 2001; Naser et al., 2004; Ryan et al., 2002; Sechi et al., 2004). Recently, Map has been cultured from the blood of Crohn's patients (Naser et al., 2004) and its presence in paraffin embedded Crohn's tissues was confirmed by IS900 in situ hybridization technique (Sechi et al., 2004). Although alternate etiologies for causation of Crohn's have been proposed, one cannot ignore the association of Map with Crohn's. A suspected route of infection may be through drinking raw or insufficiently pasteurized milk. However, Map is ubiquitous in the environment and its presence in municipal water supplies has been documented (Mishina et al., 1996). Thus, potable water could be an alternate source of infection.

Map was isolated from two-thirds of manure lagoons from herds known to be culture positive (Raizman et al., 2004) and persists in bovine feces as long as 11 months (Vishnevskii et al., 1940). Irrigation of crops with manure wastewater and fertilization with manure increases the risk of Map transmission through the consumption of contaminated fruits and vegetables.

The wide host diversity (cow, sheep, goat, deer, rabbit, mouse) of Map (Amonsin et al., 2004) also increases the likelihood of environmental contamination and spread. Map persists in the environment and is known to survive for longer than a year in inoculated grass pastures (Whittington et al., 2004). Lack of rapid and sensitive methods hitherto hindered the efforts to monitor the environmental fate and transfer of Map from animal manure to crops.

Molecular detection methods based on sequence detection of IS900, a 1451 bp repetitive DNA element (Bull et al., 2000), have been developed for the detection of Map. Many of these methods are based on the detection of amplification by end-point PCR (Jayarao et al., 2004; Marsh and Whittington, 2001; O'Mahony and Hill, 2002; Pillai and Jayarao, 2002). More sensitive real-time quantitative PCR (qPCR) methods that detect amplification during the exponential phase of amplification (Kim et al., 2002; O'Mahony and Hill, 2002) also have been reported. These methods are based on the most widely used primers designed to amplify a 229 bp (Vary et al., 1990) or a 413 bp (Millar et al., 1996) fragment of IS900. Quantitative PCR methods are based on the fluorescence detection of SYBR Green bound double stranded DNA that is formed during amplification (O'Mahony and Hill, 2002), the detection of bound fluorescent probes (molecular beacons) (Christopher-Hennings et al., 2003; Fang et al., 2002), or detection of the 5′ nuclease cleavage of a bound fluorogenic (TaqMan) probe (Kim et al., 2002) by Taq DNA polymerase. Molecular beacons and TaqMan probes are highly specific for the detection of target sequences.

The presence of 12–20 copies of IS900 in Map genome makes this unique genetic element a better target for sensitive detection as compared to the detection of single copy genes (Chui et al., 2004) like *hspX* (Stabel et al., 2004) and F57 (Coetsier et al., 2000). However, the two most popular primer sets (Millar et al., 1995; Vary et al., 1990) used to detect the IS900 region overlap regions of high homology (IS1626) with *M. avium* (Harris and Barletta, 2001). This overlap often requires additional culture steps or detection of Map specific sequences to confirm the presence of Map.

Application of molecular detection methods for detection from fecal samples often requires clean template DNA (Fus et al., 2003; Khare et al., 2004; O'Mahony and Hill, 2002; Ozbek et al., 2003; Stabel et al., 2004) necessitating elaborate DNA extraction and purification steps during which the precious template material could be easily lost. The capsular structure of Map was suspected to be inhibitory for the direct detection of cells by PCR (Fus et al., 2003) and DNA was routinely purified prior to detection by PCR. Sensitive methods that minimize loss of template DNA during processing are required to detect the low levels of Map anticipated in environmental samples.

This study focuses on developing sensitive real-time quantitative PCR methods to detect Map by using SYBR Green and TaqMan assays and to selectively differentiate Map from other closely related mycobacteria that may be present in environmental samples. Novel primers and probes targeting the amplification of short segments from different regions of IS900 were evaluated. The wide range of primers chosen allowed us to find a selective target for improved sensitivity of detection of Map. Dilutions of culture media were evaluated for the direct detection of Map cells, minimizing the loss of template DNA during extraction and cleanup, and reducing the processing time for PCR.

2. Methods

2.1. Mycobacterial cultures

Mycobacterium species and other related organisms were grown in 50 mL of 7H9 broth supplemented with 10% (vol/vol) Middlebrook OADC (Becton Dickinson, Franklin Lakes, NJ), 0.5% Tween 80 and 0.0002% (wt/vol) mycobactin J (Allied Monitor Inc., Fayette, MO) (Sung and Collins, 2000). The growth medium was supplemented with 100 μg/mL each of nalidixic acid and vancomycin, and 50 μg/mL of amphotericin B to minimize the contaminant growth during the 2–4 month long incubations. The cultures were incubated at 37 °C on a shaker at 200 rpm. Map cultures were routinely checked for purity by staining with Ziehl–Neelsen's acid fast stain or auramine–rhodamine fluorescent stain.

2.2. Extraction and quantitation of DNA

The template DNA used for real-time detection of target sequences was extracted with InstaGene™ matrix according to the manufacturer's (Bio-Rad Labs, Hercules, CA) protocol. Briefly, a 1-mL culture was centrifuged, the pellet was resuspended in 200 μL InstaGene matrix, vortexed, and incubated at 56 °C for 30 min. The samples were vortexed again, heated at 100 °C for 8 min, and then centrifuged to pellet the matrix. Aliquots of supernatants were used as template DNA without any further purification. The extracted DNA was quantitated using the PicoGreen® dsDNA quantitation kit (Molecular Probes, Inc., Eugene, OR) and by following the manufacturer's protocol. A DNA standard curve for 0–40 ng/200 μL assay volume was prepared using the lambda DNA standard provided with the PicoGreen® kit. Assays were performed in 96 well microplates and the fluorescence was measured with a SpectraMax® Gemini microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.3. SYBR Green assay

PCR reactions were carried out in 50 μL volumes containing 300 nM of each primer, 100 pg of template DNA, and 15.75 μL of 10× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) resulting in 3 mM $MgCl_2$, 200 μM each of dATP, dCTP and dGTP, 400 μM dUTP, 1.25 U AmpliTaq Gold® DNA polymerase, and 0.5 U AmpErase® uracil-*N*-glycosylase (UNG). PCR reaction mixture was prepared according to the manufacturer's protocol. An ABI PRISM® 5700 Sequence Detection System (Applied Biosystems) was used for real-time detection of amplified dsDNA with SYBR Green. Thermal cycling parameters were according to manufacturer's instructions. Briefly, AmpErase UNG incubation (50 °C for 2 min), followed by 95 °C for 10 min to activate AmpliTaq Gold, and 40 cycles of: 95 °C for 15 s and 60 °C for 1 min. Specific amplification of target DNA was monitored by comparing the normalized reporter signal (SYBR Green) for a fast threshold cycle (C_T) and the signal obtained for controls without template DNA (NTC). The determination of C_T values was according to the sequence detection system manufacturer's instructions.

2.4. Primers for SYBR Green assay

Primers targeting 50–60 bp fragments from different regions of IS900 were designed using the Primer Express software (v2.0; Applied Biosystems). Twenty-six sets of primers (Integrated DNA Technologies, Inc., Coralville, IA) with T_m 58–60 °C were evaluated at 900 nM in a 50 μL PCR mixture containing 100 pg of template DNA from Map strain ATCC 19698. Each primer set was evaluated in two replicates and the C_T

Table 1
Detection of extracted DNA from Map strain 19698 using specific primers to amplify short fragments from IS900 by SYBR Green assay

Primers[a]	Forward/reverse sequences (5′→3′)	Amplicon length, bp	C_T[b]
SF56	GGACGTCGGGTATGGCTTT	54	19.0 ± 0.1
SR109	AATCTCCTTCGGCCATCCA		
SF57	GACGTCGGGTATGGCTTTCAT	53	18.8 ± 0.4
SR109	AATCTCCTTCGGCCATCCA		
SF58	ACGTCGGGTATGGCTTTCAT	52	18.5 ± 0.2
SR109	AATCTCCTTCGGCCATCCA		
SF59	CGTCGGGTATGGCTTTCATG	51	19.0 ± 0.1
SR109	AATCTCCTTCGGCCATCCA		
SF187	TGGTCGTCTGCTGGGTTGA	53	18.6 ± 0.0
SR239	GCCACAACCACCTCCGTAAC		
SF187	TGGTCGTCTGCTGGGTTGA	54	18.6 ± 0.0
SR240	TGCCACAACCACCTCCGTA		
SF188	GGTCGTCTGCTGGGTTGATC	52	18.6 ± 0.1
SR239	GCCACAACCACCTCCGTAAC		
SF188	GGTCGTCTGCTGGGTTGATC	53	18.8 ± 0.0
SR240	TGCCACAACCACCTCCGTA		
SF189	GTCGTCTGCTGGGTTGATCTG	51	18.5 ± 0.1
SR239	GCCACAACCACCTCCGTAAC		
SF189	GTCGTCTGCTGGGTTGATCTG	52	18.5 ± 0.0
SR240	TGCCACAACCACCTCCGTA		
SF190	TCGTCTGCTGGGTTGATCTG	51	18.8 ± 0.0
SR240	TGCCACAACCACCTCCGTA		
SF199	ATGATCGAGGAGCGATTTCG	54	19.1 ± 0.0
SR252	CCAGACAGGTTGTGCCACAA		
SF200	GGTTGATCTGGACAATGACGG	53	19.1 ± 0.1
SR252	CCAGACAGGTTGTGCCACAA		
SF729	GGAACGCGCCTTCGACTAC	55	19.1 ± 0.2
SR783	GCCAGTAAGCAGGATCAGCG		
SF730	GAACGCGCCTTCGACTACAA	54	19.2 ± 0.3
SR783	GCCAGTAAGCAGGATCAGCG		
SF731	AACGCGCCTTCGACTACAAC	53	19.0 ± 0.1
SR783	GCCAGTAAGCAGGATCAGCG		
SF741	CGACTACAACAAGAGCCGTGC	57	18.1 ± 0.1
SR797	TCGGGAGTTTGGTAGCCAGTA		
SF741	CGACTACAACAAGAGCCGTGC	58	18.7 ± 0.6
SR798	GTCGGGAGTTTGGTAGCCAG		
SF742	GACTACAACAAGAGCCGTGCC	56	18.7 ± 0.0
SR797	TCGGGAGTTTGGTAGCCAGTA		
SF742	GACTACAACAAGAGCCGTGCC	57	18.7 ± 0.1
SR798	GTCGGGAGTTTGGTAGCCAG		
SF745	TACAACAAGAGCCGTGCCG	53	19.2 ± 0.2
SR797	TCGGGAGTTTGGTAGCCAGTA		
SF745	TACAACAAGAGCCGTGCCG	54	19.2 ± 0.0
SR798	GTCGGGAGTTTGGTAGCCAG		
SF965	AGGTGATGGCCCTCGACAC	61	21.3 ± 0.3
SR1025	CGAAATCGCTCCTCGATCAT		
SF1003	GCGATGATCGAGGAGCGAT	55	22.7 ± 0.4
SR1057	TGCTCAGGATGATTTCGGC		
SF1006	ATGATCGAGGAGCGATTTCG	52	19.6 ± 0.6
SR1057	TGCTCAGGATGATTTCGGC		
SF1280	CCTCCTCGCGCACCTACTAC	55	19.0 ± 0.1
SR1334	TGGGTGTGGCGTTTTCCTT		

values obtained were compared with C_T's for NTC. Optimum concentrations of selected primers were determined with combinations of 50, 300 and 900 nM of each primer in 50 μL reaction volumes containing 100 pg of template DNA from Map strain 19698.

2.5. Direct detection of Map cells as compared to extracted DNA

Ten-fold serial dilutions of cells from a 6-week-old culture of Map strain 19698 were assayed directly by the SYBR Green assay. Triplicate samples were diluted in sterile PBS (0.01 M phosphate, 0.14 M NaCl) supplemented with 0.25% Tween 80. The cell numbers used for qPCR assays were determined by direct microscopy and by plate counting. The plate counts were obtained using deep (50 mL) plates of 7H10 agar (Becton Dickinson) supplemented with or without antibiotics. The 7H10 agar contained 10% OADC and 0.0002% mycobactin J. The antibiotic supplements of 50 μg/mL each of amphotericin B, nalidixic acid, and vancomycin were added to minimize the non-mycobacterial contaminants during the 5 month long incubations at 37 °C. The plates were sealed with parafilm to minimize the drying of agar during the incubations. Purity of cells was checked by acid-fast staining. Triplicate 1-mL portions of the growth were used for extraction of template DNA with InstaGene matrix. Ten-fold serial dilutions of the InstaGene extracts were prepared in sterile ultrapure water. Five microliter portions of each of the serial dilutions of template DNA and the serial dilutions of cells were used for qPCR detection. C_T values and corresponding number of cells or the quantity of template DNA were plotted. Minimum detectable limits for template DNA or number of cells were calculated from the regression equation for the C_T value of NTC subtracted with two standard deviations.

2.6. TaqMan assays

Primers and probes were designed with Primer Express. Probes tagged with 6-carboxyfluorescein (FAM) as the 5′-reporter dye and quenched with 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end, and corresponding primers were obtained from Integrated DNA Technologies. Thermal cycling conditions for primer and probe optimizations were the same as those used for the SYBR Green assays. Probes were optimized at 25 nM increments from 25 to 225 nM. The primers were held at a constant concentration of 300 nM during probe optimizations. In addition to the primers and probe, the 50 μL of PCR reaction mixture also contained 25 μL of TaqMan® Universal Master mix (Applied Biosystems) and 100 pg of template DNA from Map strain 19698. Probes at 75 nM and primers at 300 nM were used in assays to quantify the dilutions of template DNA and to determine the specificity of the IS900-TaqMan assay to Map strains. One hundred picograms of template DNA from different species of mycobacteria was used in the specificity assays.

3. Results

3.1. Primers for IS900 detection by SYBR Green method

As the real-time PCR detection methods are highly sensitive and consistent for shorter amplicons than conventional PCR coupled with detection on gels, 26 primer sets that target 50–60 bp fragments of IS900 (Table 1) were evaluated for the detection of DNA from Map strain 19698. Except for two primer sets SF965/SR1025 and SF1003/SR1057, all primers were similarly sensitive in detecting DNA from Map strain 19698. Although they all had similar C_T values for template detection, C_T value for NTC's ranged from 30 to 38. Nearly half of the

Notes to Table 1:

[a] Forward and reverse primers are numbered based on GenBank accession number AJ250017 GI:8919133 (Bull et al., 2000). The repeat region from 492 to 1942 bp (IS900) of the 5′ end is numbered as 1 –1451 and used in the primer notations. Primer sets with low penalty scores were chosen with Primer Express. Primers evaluated at 900 nM each.

[b] Average of two replicates. One hundred picograms of template DNA from Map strain 19698 was assayed. C_T value for NTC's ranged between 30 and 38.

primer sets had C_T's for NTC ranged between 35 and 38. Two sets of primers from this batch, SF58/SR109 and SF187/SR239, were chosen for further evaluation.

3.2. Specificity of detection of Map by IS900-SYBR Green method

Map strains were differentiated from the other members of the avium complex, other species of *Mycobacterium*, and related genera (*Nocardia*, *Corynebacterium*, *Rhodococcus*) by both primer sets (Table 2). The C_T values obtained for non-Map organisms were indistinguishable or within 2–4 cycles of NTC, whereas Map strains were detected approximately 20 cycles sooner. However, variability in detection of amplified DNA was observed with different strains of Map with both primer sets evaluated. C_T values for different strains ranged between 15 and 28 for SF58/SR109 and 13 and 19 with SF187/SR239. These results suggest that primer set SF187/SR239 readily detects all of the

Table 2
Specificity of SYBR Green assay to *M. avium* subsp. *paratuberculosis*

	ATCC#[a]	Origin	Normalized threshold cycle, C_T[b]	
			SF58/SR109	SF187/SR239
Map	1038[c]	Bovine	14.8 ± 0.2	15.2 ± 0.0
Map	5016[c]	Bovine	15.3 ± 0.1	13.4 ± 0.0
Map	1040[c]	Bovine	27.8 ± 0.3	14.4 ± 0.0
Map	19698	Bovine	14.9 ± 0.1	19.1 ± 0.1
Map	KAY[c]	Bovine	18.8 ± 0.0	18.1 ± 0.1
Map	43015	Human	14.9 ± 0.3	17.1 ± 0.3
M. avium subsp. *avium*	25291	Chicken	33.4 ± 0.3	33.6 ± 0.7
M. avium subsp. *silvaticum*	49884	Wood pigeon	34.8 ± 0.8	34.0 ± 0.4
M. senegalense	35796	Bovine	34.9 ± 0.1	35.5 ± 0.2
M. bovis	19210	Bovine	34.4 ± 0.9	32.1 ± 0.1
M. vaccae	15483	Bovine	35.3 ± 0.2	35.1 ± 0.5
M. farcinogenes	35753	Bovine	NT[d]	35.0 ± 0.4
M kansasii	12478	Human	34.1 ± 0.7	36.8 ± 0.1
M. fortuitum subsp. *fortuitum*	6841	Human	33.2 ± 0.1	35.4 ± 0.2
M. smegmatis	19420	Human	34.1 ± 0.5	35.2 ± 0.1
M. intracellulare	13950	Human	34.1 ± 0.3	35.3 ± 0.3
M. tuberculosis	27294	Human	33.7 ± 0.4	34.9 ± 0.5
M. scrofulaceum	19981	Human	32.7 ± 0.3	31.7 ± 0.1
M. porcinum	33776	Swine	34.7 ± 1.2	36.5 ± 1.4
M. phlei	11758	Hay/grass	36.0 ± 1.1	35.3 ± 0.8
M. diernhoferi	19340	Dairy water trough	35.8 ± 0.4	35.3 ± 0.1
M. fallax	35219	Water	NT[d]	34.8 ± 0.1
M. chlorophenolicum	49826	Lake sediment	32.3 ± 0.0	33.2 ± 0.4
M. thermoresistibile	19527	Soil	32.0 ± 0.3	35.1 ± 0.2
M. aurum	23366	Soil	34.6 ± 1.2	38.2 ± 0.3
M. nonchromogenicum	19530	Soil	35.6 ± 0.8	34.7 ± 0.7
M. austroafricanum	33464	Soil	34.8 ± 0.5	35.2 ± 1.0
M. neoaurum	25795	Soil	33.6 ± 0.2	NT[d]
Corynebacterium bovis	7715	Bovine	37.9 ± 1.5	36.7 ± 0.5
Nocardia asteroides	19247	Human/soil	35.3 ± 1.0	34.0 ± 0.3
Rhodococcus fascians	12974	Plants	NT[d]	33.1 ± 0.3

[a] Except for the 4 Map strains from NADC[c], all other strains are ATCC type strains obtained from American Type Culture Collection (Manassas, VA).
[b] C_T values are averages of triplicates. The reaction mixture contained 100 pg of template DNA and 300 nM of each of the primers. C_T values of NTC's for primers SF58/SR109 and SF187/SR239 were 35 ± 1 and 37 ± 3, respectively.
[c] NADC strains are from the National Animal Disease Center, Ames, IA.
[d] NT—not tested.

Map strains tested, whereas primer set SF58/SR109 poorly detected strain 1040.

3.3. Sensitivity of detection of Map by IS900-SYBR Green qPCR assay

Ten-fold serial dilutions of template DNA from Map strain 19698 were assayed to determine the sensitivity of detection by two different primer sets (Fig. 1). Minimum detectable limits of 3.1 and 4.5 fg of DNA were obtained for SF58/SR109 and SF187/SR239, respectively. C_T values of NTC -2 standard deviations used in these calculations were 33.8 and 37.4 for primers SF58/SR109 and SF187/SR239, respectively (Fig. 1). Amplicons generated with SF58/SR109 were detected ~4 cycles sooner than SF187/SR239. Thus, 100 fg of template DNA was detected in 28.5 and 32.7 cycles with primers SF58/SR109 and SF187/SR239, respectively.

3.4. SYBR Green detection of cells as compared to extracted DNA

3.4.1. Cells and template DNA used for the assay

Six-week-old cultures of Map strain 19698 in 7H9 broth were used for the direct detection of cells by SYBR Green qPCR assay using primers SF187/SR239. Template DNA extracted from the same batch of cells was assayed to compare with the cell assays. Clumping of cells was minimized by including 0.5% Tween 80 in the growth medium and also by rapidly mixing the cultures during incubations. The cell growth was monitored at the end of incubations by two methods. Direct plating on deep 7H10 agar plates with and without antibiotics resulted in $1.7 \pm 0.7 \times 10^5$ and $2.2 \pm 1.0 \times 10^8$ CFU/mL. The template DNA extracted with InstaGene matrix from 1 mL of cell culture was 1120 ± 6 ng, corresponds to 5.1 fg of DNA extracted per cell. Direct plate counts from 7H10 agar without antibiotics were used for calculating the amount of DNA extracted per cell. Antibiotics in the plating medium resulted in a 3-log reduction of cell count and these counts were not used in the calculations to determine the amount of extracted DNA per cell.

3.4.2. Comparison of detection of cells and extracted DNA

Ten-fold serial dilutions of cells versus template DNA from Map strain 19698 were assayed by the SYBR Green method using primer set SF187/SR239 (Fig. 2). This primer set was chosen for its low reporter signal obtained for NTC (Fig. 1; C_T value for NTC=39.4). In the cell assay method, 5 μL of serial dilutions of cell culture in PBS–Tween 80 containing 1.1×10^{-2} to 1.1×10^6 cells was used. Serial dilutions containing less than a cell were assayed to detect the release of template DNA from cells into the medium. Serial dilutions of extracted DNA ranging from 5.6×10^{-2} to 5.6×10^6 fg were

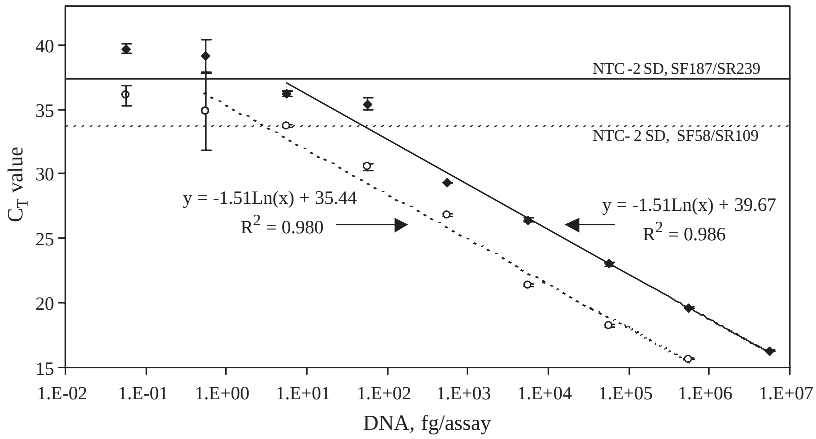

Fig. 1. SYBR Green assay of serial dilutions of DNA extracted from Map strain 19698. C_T values are plotted against femtogram of template DNA per reaction using primers SF187/SR239 (♦) and SF58/SR109 (○). Standard deviations are for triplicate assays. Lines are drawn across at C_T values 37.4 and 33.8 to show the limits of detection for primers SF187/SR239 (solid line) and SF58/SR109 (dashed line).

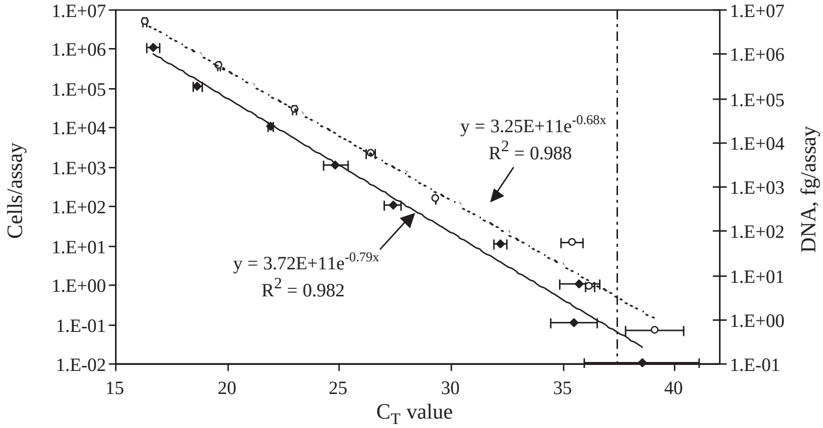

Fig. 2. Detection of cells of Map strain 19698 as compared with DNA extracted from an equivalent batch of cells by SYBR Green assay. C_T values are plotted against number of cells (◆) and femtogram template DNA (○). A primer concentration of 300 nM each of SF187 and SR239 were used in both cell and DNA assays. A dashed line at C_T value 37.4 (NTC −2 standard deviations) represents the limit of detection for both cell and DNA assays.

tested in parallel. The extracted DNA dilutions correspond to the equivalent number of cells in the direct cell assay. An inverse relationship was observed between C_T values and log cell number (R^2=0.982) and log amount of template DNA (R^2=0.988) assayed. The minimum detectable limit of the direct cell assay calculated from the regression equation was 0.07 cells. This equals 0.34 fg of extracted DNA. Detection of less than a cell was possible as the dilutions of the growth medium contained both intact and broken cells. The minimum detectable limit for the extracted DNA assays was 3.4 fg, which is calculated to equal 0.7 cell. A value of 5.1 fg of DNA extracted per cell was used in these calculations. Controls without cells or template DNA had a C_T of 39.4 ± 1.0.

3.5. qPCR detection by TaqMan method

3.5.1. Primers and probes

Three sets of primers and probes were designed and evaluated for the detection of IS900 from extracted DNA (Table 3). All three sets were equally sensitive for the detection of 100 pg of DNA from

Table 3
Primer/probe sets used in TaqMan assays

Primer/probe[a]	Forward primer/probe/reverse primer (5′ → 3′)	Amplicon length, bp	$C_T T$[b]	C_T-NTC[c]
SF30	CGTCGCTTAGGCTTCGAATT	80	22.5 ± 0.4	38.9 ± 0.9
PR72	AGCCATACCCGACGTCCCTGGG			
SR109	AATCTCCTTCGGCCATCCA			
SF53	CAGGGACGTCGGGTATGG	67	21.6 ± 0.6	37.4 ± 1.6
PR97	CCATCCAACACAGCAACCACATGAA			
SR119	GCGGGCGGCCAATC			
SF214	ATGACGGTTACGGAGGTGGTT	76	21.1 ± 0.8	>40[d]
PR265	CGACCACGCCCGCCCAGA			
SR289	TGCAGTAATGGTCGGCCTTAC			

[a] Numbering of primers and dual fluorescent probes were based on GenBank accession AJ250017, GI:8919133 (see Table 1). Probes designed for the reverse strand.
[b] C_T values for template DNA. Probes evaluated in triplicates of 6 concentrations ranging from 25 to 225 nM. Average of 18 assays.
[c] C_T values for NTC. Triplicate NTC's tested for each concentration of probe. Average C_T values for 18 NTC's.
[d] No signal for NTC during the 40 cycles of amplification.

Table 4
Selectivity of TaqMan assays for the detection of Map and related organisms

	C_T[a]
Map 19698	21.7 ± 0.1
Map 5016	17.4 ± 0.2
Map 1040	22.4 ± 0.1
Map 1038	24.9 ± 0.2
Map KAY	30.1 ± 0.1
Map 43015	19.9 ± 0.2
M. avium subsp. *silvaticum*	>40
M. avium subsp. *avium*	>40
M. intracellulare	>40
M. bovis	>40
M. tuberculosis	>40
M kansasii	>40
M. aurum	>40
M. austroafricanum	>40
M. phlei	>40
M. vaccae	>40
M. diernhoferi	>40
M. smegmatis	>40
M. thermoresistibile	>40
M. senegalense	>40
M. neoaurum	>40
M. porcinum	>40
M. fortuitum subsp. *fortuitum*	>40
M. nonchromogenicum	>40
Nocardia asteroides	>40
Corynebacterium bovis	>40

[a] Assayed with primer/probe set SF214/PR265/SR289. Reaction mixture contained 100 pg template DNA, 300 nM of each primer and 75 nM of probe. No signal detected for NTC during 40 cycles.

Map strain 19698. The primer/probe/primer set SF214/PR265/SR289 was chosen for subsequent assays as the C_T for NTC was >40 cycles and it yielded a lower C_T (21.1 ± 0.8) for the detection of Map DNA. All three probes were equally effective at all concentrations between 25 and 225 nM. Probe concentration of 75 nM was chosen for all subsequent assays to accommodate detection of high levels of template DNA.

3.6. Specificity of TaqMan assay

TaqMan assays with primer/probe/primer SF214/PR265/SR289 were specific to Map and not to any other species of mycobacteria or related genera (Table 4). DNA from other organisms gave no signal during 40 amplification cycles. Variability in C_T values was observed for the detection of same amount of template DNA from different strains of Map. It ranged between 17 and 30 cycles to detect 100 pg of template. DNA from Map strain KAY required most number of amplification cycles to detect.

3.7. Sensitivity of detection of Map by the IS900-TaqMan assay

Dilutions of extracted DNA from Map strain 19698 were assayed using primer/probe/primer SF214/PR265/SR289 and the resultant C_T's were plotted against the amount of template DNA (Fig. 3). A linear relationship was observed between C_T values and log template DNA (R^2=0.988) concentration. As signal was not detected for NTC, the minimum detectable limits were based on actual measurements. The lowest amount of detected DNA from the slope of regression

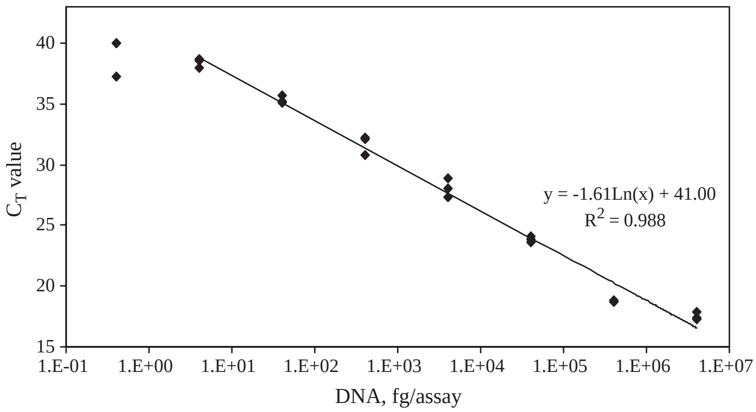

Fig. 3. Detection of template DNA from Map strain 19698 by TaqMan (◆) assay. C_T values are plotted against femtogram template DNA. The assays are with SF214/PR265/SR289 primer/probe set. The reaction mixtures contain 300 nM each of primers and 75 nM of the probe.

curve was chosen as the limit. The detection limit was 4 fg of DNA and is equivalent to 0.8 cells.

4. Discussion

With the increasing number of reports of association of Map with Crohn's patients, it is essential to determine the possible routes of transmission to susceptible individuals. One of the possible routes of transmission is through the consumption of contaminated fruits and vegetables fertilized with contaminated manure or irrigated with manure wastewater. Contamination through aerosol transport of Map to crops grown in close proximity to dairies also is a distinct possibility. Monitoring the fate, re-growth and transport of low cell numbers of Map from environmental samples requires more sensitive methods than are possible with the traditional culture methods that require 4–6 months to enumerate. In addition, the decontamination steps necessary for the gold standard culture methods are inhibitory to the growth of Map and further reduce their usefulness as methods for monitoring the environmental fate of low numbers of Map. It was our intention to develop rapid and sensitive detection methods that do not require cell culture or extensive cleanup and decontamination procedures.

Two different real-time qPCR methods based on the detection of short fragments of IS900 were developed. One of them is a general-purpose method that detects any and all SYBR Green bound amplified DNA. The second method is specific and detects the 5′ nuclease cleavage by Taq DNA polymerase of a bound fluorogenic probe during the extension cycle of amplification.

We designed 26 new primer sets that amplify short fragments (<61 bp) from different regions of IS900 for use in the SYBR Green assays. Although Map strain 19698 was detected equally well by a majority of the primer sets, different strains of Map were not detected at the same level of sensitivity by a single primer set. For example, an equivalent quantity of template DNA from strain NADC 1040 is not detected at the same level of sensitivity as other Map strains by primer set SF58/SR109. However, this strain is detected with good sensitivity by SF187/SR239. It appears from this study, multiple sets of primers may be necessary to detect the range of Map strains anticipated from environmental samples. Clearly, it is necessary to evaluate the relative sensitivity of detection of several environmental strains with a range of primer sets in order to identify the optimum assay conditions and primer sets. In addition, each fragment amplified by the 9 primer sets targeting the region of SF729 to SR798 (Table 1) has 8–9 bp mismatches as compared (CLUSTAL V) (Higgins et al., 1992) to the sequence of a new *Mycobacterium* sp strain 2333, isolated from a healthy cow (Englund et al., 2002). The genetic element of strain 2333 has 94% sequence identity to IS900. Because of the high number of mismatches, it is most unlikely that these targets would amplify. The ability of these 9 primers to differentiate Map from strain 2333 is promising and needs further scrutiny especially since the widely used Millar and Vary primers (Millar et al., 1995; Vary et al., 1990) cannot distinguish Map from strain 2333 (Englund et al., 2002). Only 1 bp mismatch, which is not in the primer region, is noted between the 229 bp fragment targeted by the Vary primers and the IS900-like sequence of strain 2333. Three mismatches were found in the 412 bp fragment amplified by Millar primers (P90/P91).

Three new primer–probe sets (Table 3) targeting amplification of short fragments (<80 bp) were identified for specific detection of Map by the IS900-TaqMan assay. The 3 primer–probe sets are equally sensitive for detecting Map strain 19698 but the sensitivity of primer–probe set SF214/PR265/SR289 resulted in different sensitivities for other strains of Map (Table 4). For example, equivalent amounts of template DNA from Map strain Kay required 8 additional amplification cycles as compared to DNA from Map strain 19698. Such variability in C_T values with strains of Map is curious and may reflect intra-species variability within the IS900 element.

The TaqMan method detects as low as 4 fg of Map specific DNA per assay and has the potential to detect even smaller quantities by increasing the amplification cycles. Increasing amplification cycles is possible as no signal for cleavage of probe is detected from controls containing only TaqMan reagents, primers, and probe. A similar detection level of 5 fg of template DNA was observed in a recently developed

TaqMan assay (Khare et al., 2004) using primers that amplify an 84 bp fragment of IS900.

The IS900-SYBR Green assay is also equally sensitive as the Taqman method, able to detect 3.1–4.5 fg of template DNA from Map strain 19698 using two different primers tested. This genetic material is equivalent to the detection of 0.6–0.9 cells. In contrast, 50 fg of DNA was necessary for detection with a SYBR Green assay developed earlier (O'Mahony and Hill, 2002) using the classic primers that amplify a 421 bp fragment (Millar et al., 1995). The higher level of sensitivity in the current study is attributed to the use of primers targeting short amplicons (<61 bp). The sensitivity of detection also depends on differentiating true amplification signal from that of SYBR Green bound to primer–dimers. Primer concentrations can be optimized to minimize the signal due to primer–dimers.

The SYBR Green assay also quantifies cells directly from Map cultures without any extractions or processing. Unlike the earlier observation of inhibition of PCR by media ingredients (O'Mahony and Hill, 2002), we found that the ingredients from 7H9 broth are not inhibitory. The method described here is highly sensitive to detect 0.07 cell per assay (equals 0.34 fg of DNA). In contrast, earlier real-time assays resulted in detection of 1–25 Map cells in pure or broth cultures (Khare et al., 2004; Kim et al., 2002; O'Mahony and Hill, 2002). Using molecular beacons, detection of 1–8 CFU by culture methods was related with the real-time detection of 0.17 fg of DNA (Fang et al., 2002). We report here for the first time a side-by-side comparison for detection of Map cells and detection of DNA extracted from equivalent numbers of cells. Cell counts from 7H10 agar plates without antibiotics were used to calculate the extracted amount of DNA per cell. We calculated an amount of 5.1 fg DNA per cell. Sanderson et al., 1992 reported 5 fg DNA per cell based on genome copy number. Based on a Map genome of 5,867,714 bp (Bannantine et al., 2002), each Map cell theoretically contains 6.8 fg of DNA. Since no extraction related losses were observed, the direct cell assay appears to be ten times more sensitive than detection using the extracted DNA approach. The limit of detection for assays using extracted DNA and primers SF187/SR239 is 3.4 fg which equals 0.7 cell. The direct cell assay also resulted in a more sensitive method than the assays using extracted DNA or with the TaqMan methods developed here and elsewhere (Kim et al., 2002).

Both methods described here are specific for the detection of Map and do not detect closely related members of the avium complex or other species of mycobacteria. Importantly, bovine, aquatic and soil mycobacteria that are common to dairy environments are well differentiated from Map. Mycobacteria of human origin (*M. intracellulare*, *M. kansasii*, *M. tuberculosis*, *M. smegmatis*, *M. scrofulaceum*, and *M. fortuitum* subsp. *fortuitum*) were also differentiated from Map.

The reporter signal observed for 100 pg of template DNA from non-Map organisms within 3 C_T's of the controls for the SYBR Green assay was suspected to make it difficult to detect low levels of Map from environmental samples. However, a comparable signal was detected with only 6.8 fg of Map DNA (value from Fig. 2 for NTC-C_T—3) which equals only 1.3 cell. As the amount of non-Map DNA assayed is equivalent to 2×10^4 cells of Map strain 19698, it is unlikely that a high number of non-Map mycobacterial cells occur in environmental samples to make a difference in the detection of low numbers of Map cells. In addition, the TaqMan assays have no amplification signal with DNA from non-Map organisms and can conclusively differentiate trace amounts of MAP DNA from that of non-MAP. Both the assays described here are simpler to use for rapid diagnostic purposes as compared to the conventional PCR panel assays developed to differentiate Map from related species (Ellingson et al., 2000).

In summary, we developed sensitive qPCR methods for detection and differentiation of Map from other mycobacteria. In addition, the direct cell assays can be easily adopted for environmental monitoring either with or without pre-enrichment and decontamination steps. Sensitive detection might alleviate some of the problems related to PCR inhibitors by direct-dilution (Ozbek et al., 2003) and yet detect low levels of Map from environmental samples. Immunomagnetic separations (Grant et al., 2000) followed by real-time sequence detection hold promise in sensitive detection of Map from environmental samples. However, these methods require validation with environmental strains of Map and real-world matrices. Although the methods are sensitive for Map specific DNA, further confirmation of viability of cells by real-time nucleic acid sequence-based amplification (NASBA) techniques (Rodriguez-Lazaro et al., 2004) is essential to monitor the fate, re-growth and transport of Map in agricultural environments.

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